INHIBITION OF PROTEOLYSIS IN MOZZARELLA CHEESE PREPARED FROM HOMOGENIZED MILK

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INTRODUCTION

Homogenization of milk was introduced almost 100 years ago to prevent the natural separation of milk phases, thus providing milk with more consumer appeal as a beverage and greater reliability and efficiency as a food ingredient (Trout, 1950). Many investigations of the physico-chemical changes that occur in milk during homogenization have been reviewed (Peters, 1964; Walstra, 1983), and there is now general agreement that the major effects are disruption of the fat globule membrane surrounding each droplet of milk fat (van Boekel and Walstra, 1989), an increase in the number of fat droplets and reduction in their size (Walstra, 1983), and formation of casein submicelles (Schmidt and Buchheim, 1970; Henstra and Schmidt, 1970). Each smaller fat droplet acquires a new "membrane" consisting of original fat globule membrane fragments complexed with casein submicelles (McPherson et al., 1984; Walstra and Oortwijn, 1982).

If cheese is manufactured from homogenized milk—for example, when recombined milk is used—the curd may lack strength and shatter (Maxcy et al., 1955; Peters, 1964); losses may occur when fine particles escape in the whey, and the final product may have poor texture characteristics (Jana and Upadhyay, 1992; Tunick et al., 1993b). Although the strong relationship between proteolysis of caseins and cheese texture is well established (Fox, 1989; Tunick et al., 1993a, 1993b), the effect of homogenization on proteolysis in cheese has rarely been investigated; this question is also related to the effects of homogenization on protein structure, a topic which also has not received much attention. Information on the effects of homogenizing cheese milk on texture and flavor in the resulting cheese could be useful in improving procedures used in the manufacture of cheese, especially low-fat cheese.

To explore the effects of homogenizing cheese milk, an experimental Mozzarella cheese developed at this laboratory was used as a model system; this cheese has very low fat levels (<10% fat by weight in the finished cheese) and high moisture content. Processing modifications introduced for the manufacture of this cheese promote casein

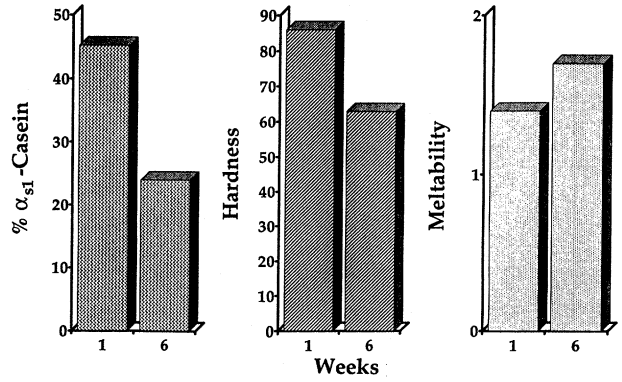

Figure 1. Correlation of α_{s1}-casein proteolysis with reduction in hardness and increase in meltability in low-fat, high-moisture Mozzarella cheese after 6 wk storage at 4° C.

breakdown, and the texture, meltability, and development of proteolysis in this cheese have been well characterized (Tunick et al., 1991, 1993a, 1993b; Malin et al., 1993). For example, Figure 1 shows the the correlation between degradation of α_{s1}-casein and the improvement of texture and meltability after six weeks of storage at 4° C (Tunick et al., 1991, 1993a, 1993b; Malin et al., 1993). In the research reported here the effects of homogenization pressure and cook temperature on proteolysis in low-fat and full-fat Mozzarella cheeses were compared. Because a lower cook temperature is used in preparing the low-fat, high-moisture Mozzarella, a standard cook temperature for some samples was included to separate temperature effects on proteolysis from those due to homogenization.

EXPERIMENTAL

Cheese Preparation

For the homogenization study, both low-fat and full-fat (referred to here as high-fat) Mozzarella cheeses were prepared on a laboratory scale (Tunick et al., 1993a, 1993b, 1994), using 22.7 kg of milk for each batch. Milk was skimmed, standardized to a specific fat content (1% for low-fat and 3% for high-fat cheeses), and pasteurized. The starter culture contained 50% *Lactobacillus bulgaricus* and 50% *Streptococcus thermophilus* (CR5 or CR7, Marschall Laboratories Division, Rhône-Poulenc,[1] Madison, WI), and the rennet was single strength (Chr. Hansen's Laboratory, Milwaukee, WI). The finished low-fat, high-moisture cheeses contained 22.3% fat in dry matter (FDM) and 57.4% moisture; corresponding values for high-fat, low-moisture cheeses were 47.6% FDM and 47.3% moisture.

The effects on proteolysis of two homogenization pressures, 10.3 and 17.2 MPa (1500 and 2500 psi, respectively) and two cook temperatures (33° and 45.9° C for low and high temperature, respectively) were investigated using a randomized 3 × 3 statistical design. It should be noted that low cook temperatures increase the moisture content, whereas low-moisture cheese results when high cook temperatures are used. Each combination of fat, moisture, and cook temperature was usually replicated two to four times. Milk for control cheeses was not homogenized; these cheeses are designated as 0 MPa.

Quantitation of Proteolysis

Cheese samples were extracted as described previously (Tunick et al.,1994); extracts were lyophilized and stored at -20° C. Samples of purified α_{s1}- and β-caseins were the gift of Dr. Elizabeth Strange of this laboratory; aliquots of α_{s1}-casein treated with chymosin and sampled hourly were provided by Dr. Paul L. H. McSweeney (University College Cork, Ireland). Casein proteolysis in cheese extracts was followed by SDS-PAGE of cheese extracts at 1, 3, and 6 weeks on 20% homogeneous PhastGels using the automated PhastSystem (Pharmacia Biotech, Piscataway, NJ); gels were stained with Coomassie blue R (Pharmacia Biotech).

To monitor the peptides formed, high-density PhastGels were also used for SDS-PAGE of cheese extracts. These 20% acrylamide gels also contain 30% ethylene glycol to retard the mobility of smaller proteins and peptides. Crosslinking with glutaraldehyde before staining was used to fix smaller peptides to the gels and prevent their disappearance; a double staining procedure (Dzandu et al., 1984), combining silver stain (Bio-Rad, Richmond, CA) and Coomassie blue R, facilitated detection of peptide bands. Low-range molecular weight markers (Promega, Madison, WI, No. V5241), ranging from 30,000 (carbonic anhydrase) to 2500 (fragment of horse hemoglobin), were used as standards.

A Bio-Rad Model 620 Video Densitomer, interfaced with a computer and Bio-Rad 1-D Analyst II software (Version 3.10), was used to scan the gels and integrate peaks. Calculations of the disappearance of α_{s1}-casein and formation of α_{s1}-I-casein were based on integrated areas of major casein bands—α_{s2}-, α_{s1}-, α_{s1}-I-, and β-caseins; smaller fragments such as *para*-κ- and γ-caseins were not included. Peptide formation was quantitated using the integrated areas of bands identified as smaller caseins and peptides, including *para*-κ- and γ_1- to γ_3-caseins. Peptides smaller than *para*-κ-casein (12,300) were calculated as a percentage of total small caseins and peptides; proteins larger than γ_1-casein (≥20,600) were not included in the calculation as major caseins cannot be resolved on high-density gels.

RESULTS AND DISCUSSION

Proteolysis of α_{s1}-Casein

Identification of α_{s1}-Casein Breakdown. Quantitation of casein proteolysis was based on breakdown of α_{s1}-casein, as this casein undergoes the major degradation in cheeses made with chymosin (Fox, 1989). In the earliest studies of the low-fat, high-moisture cheese (Tunick et al., 1991, 1993a; Malin et al., 1994), a new protein band was observed in extracts on SDS-PAGE gels between the bands of α_{s1}- and β-caseins, often at one week and always by 6 wk (Figure 2). A band for α_{s1}-I-casein was expected, as this fragment comprises residues 24-199 and is the first product of chymosin attack on α_{s1}-casein (Mulvihill and Fox, 1979; McSweeney et al., 1993a).

The major advantage of SDS-PAGE is that most globular, nonglycosylated proteins migrate at rates dependent only on molecular mass (Shapiro et al., 1967), and plots of mobility vs. log molecular weight yield straight lines enabling the estimation of molecular weight for a protein whose mobility is known (Weber and Osborn, 1969; Strange et al., 1992).

The position of the new band suggested a protein slightly smaller than α_{s1}-casein and larger than β-casein. However, Creamer and Richardson (1984) demonstrated that α_{s1}-casein does not exhibit ideal behavior; although smaller than β-casein by 10

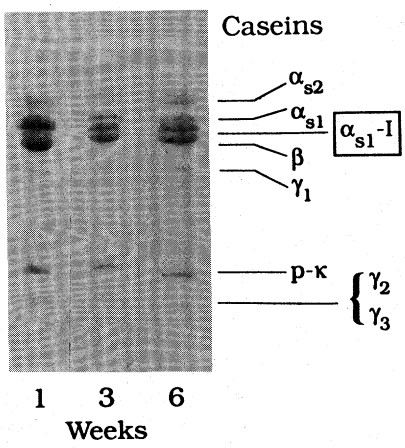

Figure 2. SDS-PAGE of extracts of low-fat, high-moisture Mozzarella cheese at 1, 3, and 6 wk. The band between α_{s1}- and β-caseins is identified in Figure 3 as α_{s1}-I-casein, resulting from the first cleavage by chymosin.

residues (equal to a mass of about 600), α_{s1}-casein migrates more slowly than β-casein. Both caseins bind SDS at the same concentration (gram/gram), but α_{s1}-casein has a greater hydrodynamic size and travels more slowly during SDS-PAGE. Because this effect was assumed to be related to the α_{s1}-casein sequence (Creamer and Richardson, 1984), there was some uncertainty regarding the identification of a new band resulting from loss of 23 residues.

Confirmation that the new band represented α_{s1}-I-casein was based on SDS-PAGE of aliquots from a kinetic study of α_{s1}-casein treated with chymosin (described above). Figure 3 shows that the first new band to appear after incubation of α_{s1}-casein with chymosin appears at a position between that of α_{s1}- and β-caseins and can therefore be identified as α_{s1}-I-casein.

Homogenization and Cook Temperature. The effects of two cook temperatures for the curd and homogenization of cheese milk at two shear pressures are shown in Figure 4. There was little difference in proteolysis of α_{s1}-casein in cheeses made from milk not homogenized (0 MPa) and milk homogenized at 10.3 MPa in low-fat cheeses cooked at low temperature (33° C). Normal breakdown kinetics were observed, as

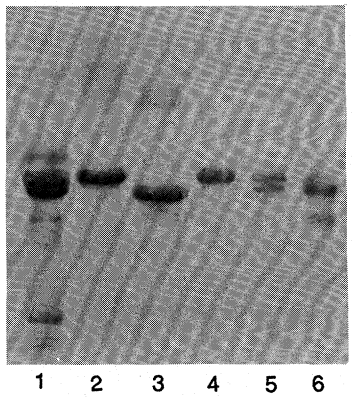

Figure 3. SDS-PAGE identification of α_{s1}-I-casein as the band observed between α_{s1}- and β-caseins. Lane 1, 6-wk extract of low-fat, high-moisture Mozzarella cheese; Lane 2, α_{s1}-casein; Lane 3, β-casein; Lanes 4-6, α_{s1}-casein treated with chymosin at 0, 1, and 3 hr.

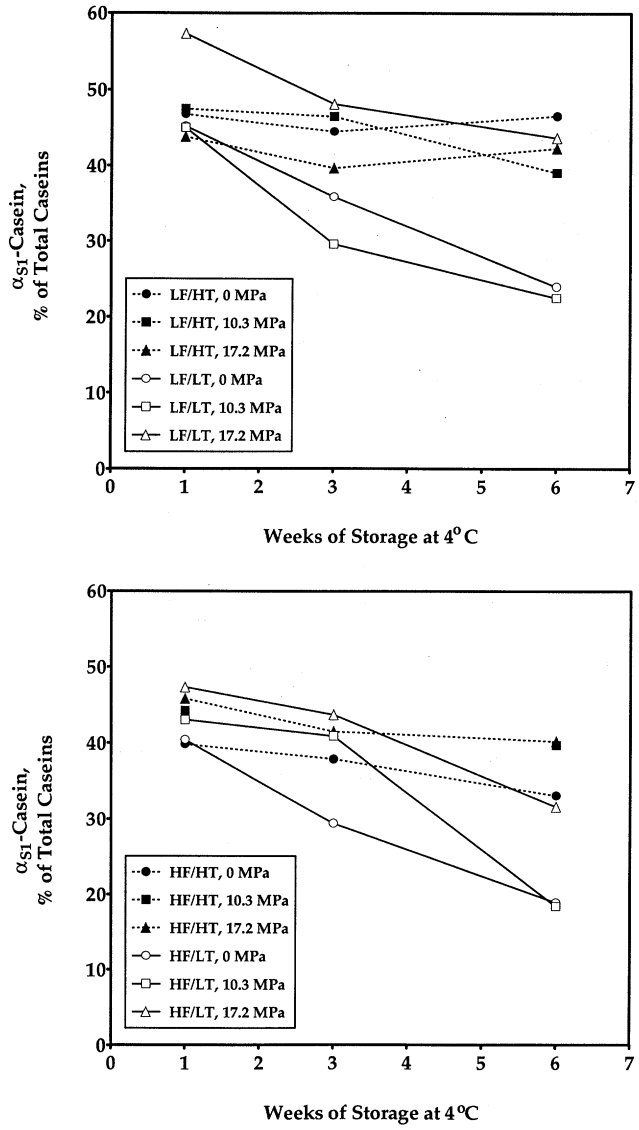

Figure 4. Effect of homogenization and cook temperature on breakdown of α_{s1}-casein in low-fat (above) and high-fat (below) Mozzarella cheeses.

exemplified by a change from 45% α_{s1}-casein at 1 week to 25% at 6 weeks. In contrast, the percentage of α_{s1}-casein in all cheeses cooked at high temperature (45.9° C) and in low-temperature cheeses prepared from milk homogenized at 17.2 MPa showed little change from the values at one week. Normal breakdown was observed for high-fat Mozzarella prepared from non-homogenized milk or cooked at low temperature, from 40% α_{s1}-casein at 1 week to 20% at 6 weeks (Figure 4). When cheese milk was homogenized at 10.3 MPa for high-fat, low-temperature cheese, the 6-week result was similar, but a lag time was indicated by the small difference between 1 and 3 weeks. Little breakdown occurred in any high-fat cheeses cooked at high temperature and in the high-fat low temperature cheese from milk homogenized at 17.2 MPa.

Formation of α_{s1}-I-Casein

The rates of α_{s1}-I-casein formation (Table 1) were similar to those of α_{s1}-casein breakdown, although some exceptions were observed. Formation of α_{s1}-I-casein is the first step in proteolysis of α_{s1}-casein by chymosin, but additional cleavage sites have been identified and breakdown continues throughout aging (Mulvihill and Fox, 1979; McSweeney et al., 1993a); a close parallel would not be expected. In low-fat, low-temperature Mozzarella cheese, rates of formation were similar for cheeses prepared from non-homogenized milk or from milk that was homogenized at 10.3 MPa; at 6 weeks the percentage of α_{s1}-I-casein averaged 23% of total caseins. As observed for the breakdown of α_{s1}-casein in low-fat cheeses, all those cooked at high temperature or prepared from milk homogenized at 17.2 MPa showed little change in α_{s1}-I-casein formation between 1 and 6 weeks, averaging 4 to 5% at 6 weeks. The α_{s1}-I-casein content of cheeses from milk homogenized at 17.2 MPa appeared to decrease from about 6% at 1 week to 4% at 6 weeks.

Rates of formation of α_{s1}-I-casein in high-fat Mozzarella cheeses (Table 1) were also similar to rates of α_{s1}-casein breakdown. Low temperatures for curd cooking or homogenization at 10.3 MPa produced similar results, and these cheeses showed approximately normal percentages of α_{s1}-I-casein at 6 weeks, about 25%. All cheeses cooked at high temperatures and those from milk homogenized at 17.2 MPa averaged one third or less of the normal values, although the cheeses from milk homogenized at 17.2 MPa averaged 12% α_{s1}-I-casein at 6 weeks.

Table 1. Effect of homogenization pressure and cook temperature on formation of α_{s1}-I-casein in low-fat and high-fat Mozzarella cheeses.

			α_{s1}-I-CASEIN, %[a]		
FAT LEVEL	COOK TEMP.	WEEKS	0 MPa	10.3 MPa	17.2 MPa
Low	Low	1	3.5	1.8	6.0
		3	11.9	15.9	5.7
		6	22.4	27.0	4.2
	High	1	1.0	0.0	0.0
		3	3.8	3.9	6.0
		6	6.8	9.9	9.9
High	Low	1	6.7	3.1	0.0
		3	18.2	6.6	1.9
		6	23.6	29.0	16.3
	High	1	3.3	2.2	0.0
		3	4.5	_[b]	0.0
		6	13.8	7.8	12.5

[a]% of total major caseins. [b]Not analyzed.

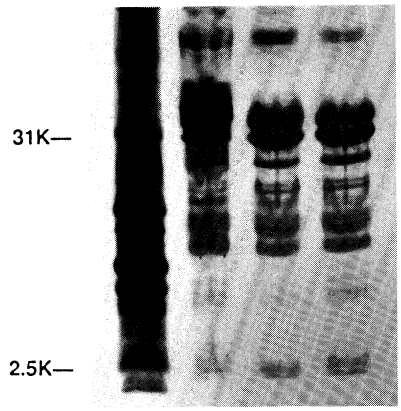

Figure 5. SDS-PAGE of 1,3, and 6-wk Mozzarella extracts on a high-density gel. The gel was treated with glutaraldehyde to prevent loss of the smallest peptides and was stained with silver-Coomassie blue to enhance visualization of bands. Left lane, standards. The range of molecular weights is indicated.

Peptide Formation

High density PhastGels provide a means for assessing peptide formation in Mozzarella cheese. Figure 5 shows a typical gel; the caseins and larger proteins appear together at the top, with peptides occupying the lower portion. The mobility of each band in the standard mixture (low molecular weight proteins and peptides) was calculated for 12 gels, averaged and plotted vs. log molecular weight (not shown). Using the mobilities of the bands in Figure 5, this plot was used to estimate the molecular weights of the observed peptide bands. Only a few of the bands could be matched to peptides predicted by chymosin and plasmin cleavage of α_{s1}-casein (McSweeney et al., 1993a, 1993b), but this may be the result of secondary protease and/or peptidase activity by starter culture enzymes, as noted by Fox et al. (see Chapter 10).

Figure 6 shows rates of peptide formation for low-fat and high-fat Mozzarella cheeses prepared from milk homogenized at 10.3 or 17.2 MPa or not homogenized at all. These data do not include the effect of temperature as a parameter—i.e., only low-fat, low-temperature and high-fat, high-temperature cheeses were studied. Rates of peptide formation were not similar to those for breakdown of α_{s1}-casein or formation of α_{s1}-I-casein. By 3 wk of storage, the rate of peptide formation had decreased and previously formed peptides were being further degraded resulting in a decline in the percentage of small peptides present in all low-fat cheeses regardless of homogenization treatment. In contrast, peptide formation had increased in all high-fat cheeses, and this also was independent of homogenization. By 6 wk, the patterns were somewhat reversed. Small peptide formation in the low-fat cheeses had increased to 1 wk values or greater, and in the high-fat cheeses, peptide formation had stabilized or decreased.

The explanation for these data may lie in the prolonged survival of starter culture bacteria in the low-temperature cheeses. Low-temperature cooking, as defined earlier in the Experimental section, leads to a higher moisture content in the cheeses and, coupled with the sparing effect of the lower temperature itself, prolongs the survival of starter culture bacteria. Scanning electron micrographs of both low-fat, high-moisture (low temperature) and high-fat, low-moisture (high temperature) Mozzarella cheeses show 50% greater survival of both *L. bulgaricus* and *S. thermophilus* in the low-fat, high-moisture cheese (Tunick, et al., 1993a). Because the starter culture microorganisms probably release their proteases and peptidases only upon death and autolysis, most of the casein breakdown after low-temperature cooking would be confined to cleavages by chymosin and plasmin until bacteria began to die, and the percentage of peptides would be low at early times. Conversely, high cook

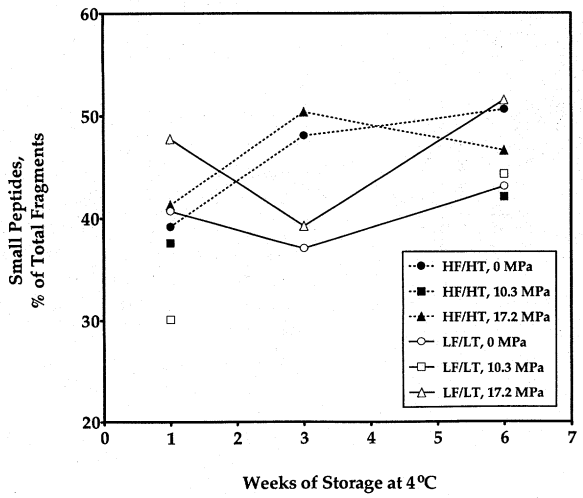

Figure 6. Effect of homogenization on peptide formation in low-fat, high-moisture and high-fat, low-moisture Mozzarella cheeses.

temperatures would permit survival of fewer starter bacteria. Their death and autolysis would be expected to result in the increase in peptide formation shown in Figure 6 at 3 wk, and increases after that would not be likely.

CONCLUSIONS

Homogenization of cheese milk at 10.3 MPa had little effect on proteolysis in either low-fat or high-fat Mozzarella cheese cooked at low temperature. Low cook temperatures promote survival of chymosin, plasmin, and starter culture bacteria, and α_{s1}-casein comprised 20 to 25% of total major caseins for both types of low-temperature cheese by 6 wk. However, proteolysis of α_{s1}-casein and formation of α_{s1}-I-casein in low-fat and high-fat cheese was retarded by homogenization of cheese milk at 17.2 MPa or by higher cook temperature. The 6 wk values for α_{s1}-casein in high temperature or 17.2 MPa cheeses were 40-47% and 34-42% for low- and high-fat cheeses, respectively. Homogenization did not affect rates for small peptide formation; rather, moisture levels resulting from low or high cook temperatures were primarily responsible for the observed results.

The effects of cook temperature and the 17.2 MPa homogenization pressure resulted in similar outcomes for α_{s1}-casein breakdown or α_{s1}-I-casein formation, but different mechanisms appear to be responsible. High cook temperature has a major role in retarding proteolysis because it leads to lower moisture in the cheese; each peptide bond undergoing proteolysis requires a molecule of water, so moisture content is critical. Higher cook temperatures may also inactivate chymosin, at least partially. Homogenization at 17.2 MPa in cheeses cooked at low temperature obviously induces changes that reduce breakdown of α_{s1}-casein, especially in low-fat cheeses. These alterations could be changes in protein conformation that obscure cleavage sites of α_{s1}-casein or alter the local environments of these sites, effectively reducing the enzymatic activity of chymosin with respect to α_{s1}-casein. Preliminary studies have suggested that homogenization induces covalent disulfide linkages between β-lactoglobulin and κ-casein (Holsinger, et al., 1992), and similar linkages are induced by heating (Parris, et al., 1990). The presence of crosslinked β-lactoglobulin and κ-casein fragments might,

therefore, also have an effect on proteolysis. Future studies relating homogenization and the structure of cheese proteins are expected to clarify the effects of homogenization in retarding proteolysis.

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